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POSITIONAL IDENTIFICATION AND FUNCTIONAL ANALYSIS OF GENES REGULATING AUTOIMMUNE ARTHRITIS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The major histocompatibility complex (MHC) is the most gene-dense and polymorphic region in the human genome with strong associations to many autoimmune disorders, including rheumatoid arthritis (RA). However, even the genetic association between MHC and RA was known more than 40 years ago, we still have not fully explained the functional roles of the MHC genes and identified the underlying specific polymorphisms. This thesis describes some of our research aimed for a better understanding of this topic, which can largely be divided into three parts as follows.

First, we made use of a panel of MHC class II (MHC-II) congenic strains to evaluate the functional roles of MHC-II polymorphisms in arthritis. By performing an extensive genetic and functional analysis, we showed that MHC-II *RT1-B* (the rat orthologs of *HLA-DQ*) determines the onset and severity of experimental arthritis, possibly due to the amino acid variations in the P1 pocket of RT1-B. In addition, we showed that natural allelic variants in *Tap2*, another gene in the MHC-II region, regulates the thymic selection of CD8+ T cells.

Second, in order to investigate whether other MHC genes also contribute to arthritis susceptibility, we assessed arthritis development in congenic strains mapped to other parts of the MHC region. We identified a second arthritis-regulatory QTL in the MHC class III region, that regulates not only the onset and severity, but also chronicity of arthritis. We subsequently mapped this effect to a conserved, 33-kb large haplotype *Ltab-Ncr3* comprising five polymorphic genes. Interestingly, unlike other positionally-identified arthritis genes in rats, *Ltab-Ncr3* regulates only adjuvant arthritis models but not autoimmunity triggered by specific tissue antigens, such as type II collagen. Furthermore, we found that gene expression and alternative splicing of the *Ltab-Ncr3* genes correlate remarkably with arthritis severity and some of the gene expression differences were reproduced in a cohort of RA patients and healthy controls.

Third, the MHC-II gene expression is regulated by class II transactivator (CIITA or C2TA), and in humans, genetic variation in *CIITA* has been associated with differential expression of MHC-II and susceptibility to autoimmune diseases. Using a congenic mouse strain with an allelic variant in the type I promoter of *C2ta*, we demonstrate that whereas genetic polymorphisms in *C2ta* promoter result in differential MHC-II expression and antigen presentation, these do not necessarily have a strong impact on autoimmune diseases such as arthritis.

In summary, these studies demonstrate how the congenic approach remains powerful to conclusively identify and characterise genes regulating a complex disease like arthritis.

LIST OF SCIENTIFIC PAPERS

I. **Positional identification of *RT1-B* (*HLA-DQ*) as susceptibility locus for autoimmune arthritis**
Sabrina Haag*, Jonatan Tuncel*, Soley Thordardottir, Daniel E. Mason, Anthony C. Y. Yau, Doreen Dobritzsch, Johan Bäcklund, Eric C. Peters, & Rikard Holmdahl
J Immunol. 2015 Mar 15;194(6):2539-50

II. **Class II major histocompatibility complex–associated response to type XI collagen regulates the development of chronic arthritis in rats**
Jonatan Tuncel, Sabrina Haag, Stefan Carlsén, Anthony C. Y. Yau, Shemin Lu, Harald Burkhardt, Rikard Holmdahl
Arthritis Rheum. 2012 Aug;64(8):2537-47

III. **Natural polymorphisms in *Tap2* influence negative selection and CD4:CD8 lineage commitment in the rat**
Jonatan Tuncel, Sabrina Haag, Anthony C. Y. Yau, Ulrika Norin, Amelie Baud, Erik Lönnblom, Klio Maratou, A Jimmy Ytterberg, Diana Ekman, Soley Thordardottir, Martina Johannesson, Alan Gillet, EURATRANS Consortium, Pernilla Stridh, Maja Jagodic, Tomas Olsson, Alberto Fernandez-Teruel, Roman A. Zubarev, Richard Mott, Timothy J Aitman, Jonathan Flint, Rikard Holmdahl
PLoS Genet. 2014 Feb 20;10(2):e1004151

IV. **Conserved 33-kb haplotype in the MHC class III region regulates chronic arthritis**
Anthony C. Y. Yau, Jonatan Tuncel, Sabrina Haag, Ulrika Norin, Miranda Houtman, Leonid Padyukov, Rikard Holmdahl
Proc Natl Acad Sci U S A. 2016 Jun 28;113(26):E3716-24

V. **MHC class III *Ltab-Ncr3* haplotype regulates adjuvant-induced but not antigen-dependent autoimmunity**
Anthony C. Y. Yau, Jonatan Tuncel, Rikard Holmdahl
Manuscript (Under Revision)

VI. **Effects of *C2ta* genetic polymorphisms on MHC class II expression and autoimmune diseases.**
Anthony C. Y. Yau, Fredrik Piehl, Tomas Olsson, Rikard Holmdahl
Manuscript (Under Revision)

* These authors contributed equally.

CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti–citrullinated protein antibodies
AIL	Advanced intercross line
APC	Antigen-presenting cells
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CIITA (or C2ta)	Class II transactivator
CII	Collagen type II
CD	Cluster of differentiation
DMARD	Disease modifying anti-rheumatic drug
EAE	Experimental autoimmune encephalomyelitis
G6PI	Glucose-6-phosphate isomerase
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HS	Heterogeneous stock
IFA	Incomplete Freund's adjuvant
IL	Interleukin
LD	Linkage disequilibrium
LST1	Leukocyte specific transcript 1
LTA	Lymphotoxin alpha
LTB	Lymphotoxin beta
Mbt-AIA	Mycobacterial adjuvant-induced arthritis
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MTX	Methotrexate
NCF1	Neutrophil cytosolic factor 1
NCR3	Natural cytotoxicity triggering receptor 3
OIA	Oil-induced arthritis
PIA	Pristane-induced arthritis
QTL	Quantitative trait locus

RA	Rheumatoid arthritis
RF	Rheumatoid factor
rSCH	Rat spinal cord homogenate
SE	Shared epitope
SNP	Single nucleotide polymorphism
TNF	Tumour necrosis factor

1. RHEUMATOID ARTHRITIS

Rheumatoid Arthritis (RA) is a chronic, autoimmune disorder characterised by the destruction of synovial joints, leading to joint deformity and severe disability. It is estimated to affect around 0.5-1.0% of adults in the developed world with a higher prevalence in women than men, and at older ages (1). Major symptoms of RA are pain, stiffness and swelling of multiple peripheral joints. Uncontrolled active RA not only leads to reduced quality of life, but also a shorter life expectancy because of cardiovascular and other comorbidities. RA also leads to enormous direct and indirect costs to society. For instance, around one-quarter of individuals with RA, if untreated, will not be able to work within two to three years of clinical diagnosis (2). Both genetic and environmental factors contribute to the development of the disease. So far more than 100 risk loci, as well as several environmental risk factors have been linked to RA (3, 4). Despite the enormous progress made in understanding this complex disease, especially over the past twenty or so years, there is currently no preventive treatment or cure for RA. Thus, gaining a better knowledge into the aetiology of RA is essential for developing effective therapies to cure this disease. Animal models have proven to be important to study the cause, development and treatment of the disease.

1.1 Autoimmunity

Based on our current epidemiologic, genetic and clinical findings, RA pathogenic development can be largely divided into three stages, autoimmune priming, tissue attack and chronic inflammation (5). In the first stage, environmental factors seem to trigger innate immunity and induce adaptive immune responses in genetic-susceptible individuals, leading to autoantibody production and disease development at a later stage. During this stage, autoantibodies, such as rheumatoid factors (RFs) (6), anti–citrullinated protein antibodies (ACPAs) (7) can be detected in serum. Epidemiological data suggested that environmental factors, rather than MHC-II genetic association, drives the early production of autoantibodies suggesting a role of innate immune system at this stage (8). Genetic factors such as MHC-II alleles may have a more important role in determining the susceptibility of developing RA (8, 9). It is not exactly known which adjuvants triggers disease onset in genetically-susceptible individuals but mineral oil exposure and cigarette smoking have been associated with ACPA and RF responses in RA (9–11). The second stage is the clinical onset marked by a joint-specific inflammatory reaction. An increased number of activated $CD8^+$ T cells and $CD19^+$ B cells was found in inguinal lymph nodes (12) and ACPAs have been shown to be able to stimulate osteoclastogenesis causing structural bone damage even before clinical onset (13). However, whether autoreactive T and B cells, and autoantibodies such as RFs and ACPAs are arthritogenic or regulatory in pre-arthritis phase is currently not clear. The third stage is when the disease develops into an active, chronic relapsing inflammation leading to tissue destruction and bone deformity. The wide range of effective biological treatments indicates the heterogeneity of the disease. For example, chronic RA can be treated by neutralising TNF and IL-6 (which is largely produced by macrophages), by blocking T-cell costimulation or by anti-CD20 treatment as discussed further in section 1.7.

1.2. Epidemiology

The incidence of RA increases with age. In Sweden, the mean age of onset is around 50-60 years old with a female dominance (7). The lifetime risk of developing RA among adults is estimated to be around 2.7% for women and 1.5% for men. Globally, there is regional variation in the prevalence of RA (14, 15). The native American-Indian populations have the highest recorded prevalence of RA, 5.3% for Pima Indians and 6.8% for Chippewa Indians. In contrast, lower prevalence has been found in Africa and Asia with a prevalence of 0.2-0.3% reported in China and Japan (14, 15). A prevalence of 0.5-1.1% has been found in Northern Europeans and North Americans; and 0.3-0.7% in Southern Europeans. This regional variance in RA prevalence could be due to both genetic and environmental factors.

1.3. Diagnosis Criteria

The diagnosis of RA depends on clinical features, laboratory tests and/or radiological testing. One of the most widely accepted criteria for defining RA is the American College of Rheumatology (ACR) 1987 criteria (16) (Table 1), which is designed to identify patients with established RA and exclude those with other rheumatological diagnoses (16). However, the ACR 1987 criteria have been criticized for their lack of sensitivity to identify patients who could benefit from early effective intervention. Therefore, ACR and European League Against Rheumatism (EULAR) developed the 2010 ACR/EULAR classification criteria for RA with the main goal of facilitating the identification of individuals at the earlier stages of the disease (17) (Table 2). In addition to RF already included in the 1987 ACR criteria, indicators of ongoing inflammation including ACPA, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), are included in the 2010 classification criteria.

Table 1: The 1987 ACR criteria for the classification of RA (16)

I.	Morning stiffness Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
II.	Arthritis of 3 or more joint areas At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
III.	Arthritis of hand joints At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
IV.	Symmetric arthritis Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
V.	Rheumatoid nodules Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
VI.	Serum rheumatoid factor Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
VII.	Radiographic changes Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localised in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify).

Classification criteria: A patient shall be said to have RA when four or more of the seven criteria are fulfilled. Criteria I - IV must have been present for at least 6 weeks. PIP: "proximal interphalangeal joints, MCP: metacarpophalangeal joints, MTP: metatarsophalangeal joints.

Table 2: The 2010 ACR/EULAR criteria for the classification of RA (17)

I.	Joint involvement	
	1 large joint	0
	2–10 large joints	1
	1–3 small joints (with or without involvement of large joints)	2
	4-10 small joints (with or without involvement of large joints)	3
	>10 joints (at least one small joint)	5
II.	Serology (at least 1 test result is needed for classification)	
	Negative RF and negative ACPA	0
	Low-positive RF or low-positive ACPA	2
	High-positive RF or high-positive ACPA	3
III.	Acute-phase reactants (at least one test result is needed for classification)	
	Normal CRP and normal ESR 0	0
	Abnormal CRP or normal ESR 1	1
IV.	D. Duration of symptoms	
	<6 weeks	0
	≥6 weeks	1

Classification criteria: total score of criteria I – IV and a score of ≥ 6/10 is needed for classification of a patient as having definite RA.

1.4 Autoantibodies

RA patients have autoantibodies against a variety of potential autoantigens, including fibrinogen, vimentin, collagen and α-enolase, that display varying sensitivity and specificity in RA. One of the 1987 ACR criteria and the 2010 ACR/EULAR criteria for the classification of RA is serology (16, 17). It is known that autoantibodies, such as RF or ACPA, can be detected in serum many years before the clinical onset (7, 18, 19), although the exact time point they appear remain to be clarified. The response to treatment has also been associated with the presence of RF and ACPA (20).

RF is an autoantibody against the Fc portion of IgG antibodies. RF is primarily associated with RA, present in around 75% of patients with RA (21) and can be detected many years before RA onset (6, 7). The presence of RF in patients with RA has also been linked to severity and increased mortality (22, 23). However, the specificity of RF is low and can be detected in patients with other autoimmune disorders and infectious disease (24), as well as 3-5% of healthy population. ACPAs, on the other hand, are more specific for distinguishing RA from other rheumatic diseases and are more predictive for future development of RA (25, 26), and are therefore a better diagnostic and predictive marker of RA. ACPAs were first discovered in 1964 as anti-perinuclear factor (APF) directed to keratohyaline graunules in the cytoplasm of buccal mucosal cells (27). APF antibodies were found to be highly sensitive (present in 49-91% of RA patients) and highly specific (73-99%) (28). It was later found that the recognition of antigen depended on the process of citrullination, which

converts arginine to citrulline by the enzyme peptidylarginine deiminase (29). Based on these findings, commercial assays have been developed to test for the presence of autoantibodies to cyclic citrullinated proteins (CCP).

1.5 Genetic factors

RA is a genetic disease and this is evident from the concordance rate of 15% among monozygotic twins (30, 31). The heritability of RA is estimated to be 40-60% (8, 30, 32). ACPA-positive and ACPA-negative RA have been described as two genetically different subsets, since they differ in susceptibility loci, the effect size, as well as the strength of genetic association (33).

Among all the RA risk loci identified, the human leukocyte antigen (HLA), encoded by the major histocompatibility complex (MHC), exhibits the strongest association to RA. The HLA region was linked to RA half a century ago (34), long before the GWAS was performed. In humans, the MHC locus spans around 4 Mb containing over 200 genes; around half of these genes have immune-related functions. The MHC region is characterised by high gene density, extreme polymorphism and strong linkage disequilibrium (LD), which has hampered the identification of variants driving the MHC disease association. In fact, the MHC region has been described as the most gene-dense in the human genome (35). The MHC was first identified to be associated with RA when a higher prevalence of HLA-DR4 was found in mixed lymphocyte cultures of RA patients (34). The 'shared-epitope' hypothesis later described a conserved amino acid sequence motif comprising residues 70-74 in the third hypervariable region in the HLA-DRB1 chain that is associated with RA (36). The shared epitope (SE) association was recently refined to six different amino acids in HLA-DRB1, HLA-A, HLA-B, and HLA-DPB1 (3, 4).

Association with several other non-HLA genes were discovered after MHC association, including *PTPN22* (*protein tyrosine phosphatase 22*) (37) and *CTLA4* (*cytotoxic T-lymphocyte protein 4*) (38), *PADI4* (*protein-arginine deiminase type 4*) (39), *TRAF1* (*tumour necrosis factor receptor-associated factor 1*) (40) and *FCRL3* (*Fc receptor-like protein 3*) (41). Most other risk loci we know today are identified in GWAS and they confer relatively modest effects (OR < 1.2). It is important to note that despite enormous progress made over the past 20 years in identification of risk loci, all the risk loci we have identified so far only explain a small fraction of the genetic variance of RA. HLA explains the ~13% of the genetic variance; and other non-MHC loci explain another 5% (3, 4) meaning a significant proportion of genetic variance of RA remains unexplained. There are multiple reasons that we have not been able to identify this 'missing heritability'. These include genetic heterogeneity, the low penetrance of disease alleles, strong LD with nearby genes, as well as gene-gene and gene-environmental interactions taking place which hamper the identification of disease-driving genetic factors (42).

1.6 Environmental factors

While the exact pathogenic mechanisms triggering RA is unclear, different environmental triggers have been suggested including smoking, exposure to silica and oil, alcohol, diet, oral bacteria, gut microbiota, and socioeconomic status, as discussed below.

Smoking is by far shown to be the strongest risk factor for the development of RA and consistently replicated in different populations (43–48). Any type of smoking (ever, current and past) increases risk for developing RA (49). The risk of developing RA is dose-dependent and increases by 26% for 1-10 pack-years, 94% for 20-30 pack-years and 107% for >40 pack-years (50). This risk was significantly higher among RF-positive RA cases (49, 50). The effect of smoking on RA is long-lasting and risk remains elevated 20 years or more after cessation (43, 51), although one study found that such risk decreased over time since cessation (43). Cigarette contains more than 4000 chemical compounds (including over 50 known carcinogens), which may have an adjuvant effect as discussed later.

The association of the risk of developing RA with cigarette smoking has led to further studies into any RA association in respiratory exposure to air pollutants, with some evidence suggesting that exposure to air pollution could increase risk of RA (52, 53). Occupational exposure to silica through exposure to stone dust, rock drilling or stone crushing was also associated with an increased risk of developing RA in Caucasian and Asian populations (54, 55). An increased risk of developing RA was also found in workers exposed to mineral oils (10). It is known that administration of adjuvant induces an erosive polyarthritis in certain rat strains (56, 57). Whether similar mechanisms also operate in humans is an open question and animal models could be useful in understanding how adjuvants may trigger autoimmunity in humans.

Interestingly, a significant inverse association was found between low to moderate alcohol consumption and RA risk for both men and women (58–61). Compared with non-drinkers, low to moderate alcohol consumptions reduces risk for developing RA in a manner that appears to be dose-dependent, time dependent and sex dependent. However, as in most epidemiological studies, such studies can be vulnerable to influences from confounding factors, for instance, underestimation or underreporting in alcohol consumptions by the participants.

Dietary habits have been studied for association with risk of developing RA. For instance, fatty fish consumption has been associated with disease protection (62–65). Greater intake of fruits and (cruciferous) vegetables, as well as supplemental vitamins, and lower intake of red meat could reduce RA risk (66–68). The traditional Mediterranean diet featured with high consumption of fruit, vegetables, fish, olive oil, less red meat and moderate alcohol has been also associated with protective effects in some diseases (69) but not RA (70). However, these data should also be taken with cautions since not all the data could be reproduced and some are inconclusive due to low number of RA patients (65, 71–73).

Higher disease prevalence in women suggests that hormones could play a role in arthritis development. This is consistent with the findings of reduced disease onset during pregnancy and increased risk of developing RA postpartum (74). RA activity was also found to reduce during pregnancy and ‘relapse’ after delivery (75, 76). Oral contraceptive

use protects patients with definite RA from progression from mild to severe disease (77, 78) and hormone replacement therapy brings improvement in inflammation (79). Similar protective effect of female sex hormone was shown in both collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) in rodents (80–82).

In 1982, Snyderman and McCarty first proposed a correlation between RA and adult periodontitis. Both diseases are characterised by self-sustaining inflammation around bone and subsequent bone erosion. Patients with long-standing active RA are more likely to develop periodontal disease, and vice versa (83–85). Elevated level of oral anaerobic bacterial antibodies could be found in the serum and synovial fluid of patients with RA (86–88). It is hypothesised that one of the oral pathogens, *Porphyromonas gingivalis*, expresses a unique microbial enzyme, peptidylarginine deiminase (PAD), that citrullinates proteins, including fibrinogen and α-enolase, which are major potential autoantigens in RA and lead to intraarticular inflammation (84). This hypothesis is supported by an increase in anti-*P. gingivalis* antibody levels in RA patients (89, 90), which developed years before onset of symptoms (91). Presence of periodontal bacteria in synovial joints has been associated with an exacerbated CIA in mice (92). Furthermore, comparative studies conducted on faecal samples from patients with RA found changes in microbial species, diversity and abundance compared with controls (93–96) and altered composition of human intestinal microbiota has been suggested to contribute to arthritis development (97). While the mechanistic basis of etiological link between RA and bacteria is not completely understood, current data suggest that they may play a role in the regulation of autoimmune arthritis.

1.7 Treatment

The main goal of RA treatment is to stop inflammation, relieve symptoms, prevent joint damage, improve mobility and reduce long-term implications. Lessons from multiple clinical trials in treating early RA patients has led to the current recommendations that treatment should aim at the early stage of the disease course with intensive intervention to target remission.

The current first line of treatment is disease-modifying anti-rheumatic drugs (DMARDs), used either alone or in combination with other drugs. Among the different DMARDs available, methotrexate (MTX) is well-known for its long-term safety and clinical efficacy and is recommended by EULAR as first line treatment for patients with active RA (98). Other frequently used DMARDs include sulfasalazine, hydroxychloroquine, and leflunomide. Less common medications are gold salts, azathioprine, and cyclosporine.

With the introduction of biological agents such as tumour necrosis factor (TNF) inhibitors (inflixmab, etanercept, adalimumab, certolizumab and golimumab), anti-CD20 (rituximab), CTLA-4Ig (abatacept), anti-IL-6R (tocilizumab) and anti-IL-1RA (anakinra), there has been both significant reduction in disease activity and improvement in quality of life in RA patients who receive treatment. In particular, many studies demonstrated that RA patients benefited from TNF inhibition treatment with reduced disease activity (99). In 2015, the ACR issued recommendations on how patients not responding to DMARDs should be

treated with a TNF inhibitor alone or in combination with other DMARDs (100). There have been reports that etanercept treatment led to higher incidence of serious infections and malignancies although these have not been proven (101, 102).

For patients not responding to MTX or other synthetic DMARDs, Tocilizumab, which is a humanised monoclonal antibody against IL-6 receptor, has proven to be effective (103, 104), and can be used alone or in combination of MTX or other sDMARDs (105–107). Abatacept is a fusion protein composed of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the Fc portion of IgG1. CTLA-4Ig binds to CD80 and CD86 on APCs, blocks CD28 engagement and thus prevents T-cell activation (108). Early RA patients treated with Abatacept and MTX achieved better clinical outcomes than patients treated with MTX alone (109, 110). Rituximab is a chimeric monoclonal antibody targeting CD20, a protein expressed on B cells. It acts by depleting B cells and is used in patients not responding to other biologics. When used with MTX, it leads to significantly less synovitis and reduced erosion and cartilage loss for responding patients (111, 112).

Nevertheless, almost 20-50% of patients do not adequately respond or have to discontinue the use of conventional and biological DMARDs due to intolerance or other adverse events, prompting the development of several other new drugs (113). One of them is Tofacitinib, which acts by inhibiting the enzyme janus kinase 1 (JAK1) and JAK3 in the intracellular signal transduction of many cytokines. Other drugs, such as Peficitinib and Decernotinib, bind to different members of the tyrosine kinase family. Tabalumab is an antibody that neutralises both soluble and membrane-bound B-cell activating factor (BAFF). Atacicept is a recombinant fusion protein that neutralises the activity of APRIL and BLyS (B lymphocyte stimulator). Brodalumab (IL-17 receptor), Denosumab (RANKL) and Mavrilimumab (GM-CSF) are other antibodies under development.

2. ANIMAL MODELS OF RHEUMATOID ARTHRITIS

Animal models for RA are essential not only to advance our understanding of the pathogenesis of RA, but also to develop treatments for a disease that is still affecting millions of people across the world.

Firstly, animal models are important to improve our understanding of genetic contributing factors of RA. As previously mentioned, the >100 risk loci so far identified in fact account for only a modest fraction of the genetic variance associated with the disease (3, 42); and for most of these loci, very little is known about their pathogenic roles. Human genetic studies are restrained by factors such as the genetic heterogeneity, strong LD with nearby genes, small effect size of risk loci, as well as the possibility of gene-gene and gene-environment interactions involved in the regulation of this complex disease. Animal models of RA overcome some of these limitations and are therefore attractive alternative approaches to human genetic studies to identify causative genes and to understand the underlying pathogenic mechanisms. For instance, the possibility to perform selective interbreeding of inbred lines followed by disease phenotyping of the offsprings, allows the identification of quantitative trait loci (QTLs) that regulate arthritis. A quantitative trait locus is defined as a chromosomal region linked to a variation in a phenotype (which can be a disease phenotype). The possibility to isolate the QTL in the form of congenic fragments and subsequent minimizing the size of the congenic fragment allows for positionally cloning and deep analysis of the gene(s) that regulates the disease trait. This strategy has so far proven to be successful to identify and characterise novel disease genes as will be discussed later. Secondly, animal models are optimal to study the environmental influence on RA, including smoking, alcohol, diet, and bacterial infections. In a laboratory setting, such factors can be carefully studied in a controlled way. Lastly, animal models are needed to firstly develop better drugs for the treatment of RA and for testing both their effectiveness and safety. Current European legislations require toxicity tests of any drugs in at least two mammalian species before being licensed for human use.

2.1. Rat Models of arthritis

In order to use animal models to improve our understanding of both genetic and environmental causes of RA, as well as to develop effective treatments for RA, well-characterised animal models that reflect the complex nature of the disease are necessary. Since the work presented in this thesis is mostly based on rat congenic strains, rat models of RA will be the main focus of this discussion. In rats, there are mainly two categories of induced arthritis models. The first category is based on disease induction by cartilage-derived proteins, as exemplified by collagen-induced arthritis (CIA) (114). The second category is based on disease induction by adjuvants alone, as exemplified by pristane-induced arthritis (PIA) (115, 116) and oil-induced arthritis (117).

2.1.1 Collagen-induced arthritis (CIA)

CIA is one of the most commonly used models of RA in both rats and mice. Different cartilage derived proteins, including type II collagen (CII) (114), type XI collagen (CXI)

(118), and cartilage oligometric matrix protein (COMP) (119) can be used to induce arthritis in rats. CIA is typically induced by intradermal injection of native autologous rat CII, emulsified in incomplete Freund's adjuvant (IFA) at the base of the tail (120). As opposed to CIA in mice, no mycobacterial addition or booster injection is needed in rat CIA. The disease course in rats is more rapid than in mice. The typical onset is around 12 to 14 days after immunisation when rats of the DA strain start to develop polyarthritis. The disease then enters into a phase of remission before a chronic relapsing phase (121). Both B cells reactive towards native CII epitopes as well as autoreactive T cells induced by immunisation with autologous CII are important for the development of CIA (121, 122). The adoptive transfer of CIA by CII-reactive T cells is not as effective as in arthritis induced by adjuvants such as pristane or IFA (123, 124), suggesting the dependency on both B cells and T cells in CIA in rats. One advantage of CIA over mycobacterial adjuvant-induced arthritis (Mbt-AIA) (section 2.1.5) is that the former is driven by autoimmune response to a defined connective tissue component rather than a bacterial arthritogen. CIA shares many clinical and subclinical features of human RA, including the dependency on both MHC and non-MHC genes, production of anti-collagen antibodies, an inflammatory process primarily directed to peripheral joints and a chronic disease course (125–128).

2.1.2 Pristane-induced arthritis (PIA)

PIA is another commonly used rat model of RA. PIA is induced by an intradermal injection of pristane at the base of the tail, leading to a chronic relapsing disease course (115, 116). Pristane (2, 6, 10, 14-tetramethylpentadecane) is a saturated hydrocarbon with 15 carbons on its backbone and four methyl groups at position 2, 6, 10 and 14. Pristane is a natural component of chlorophyll and is therefore normally eaten and absorbed by animals. Thus PIA, unlike CIA, is independent of exogenous antigens and it is interesting that a single injection of this 'self' component in susceptible rat strain can lead to chronic inflammatory disease. The subcutaneous or intradermal induction of arthritis by pristane is unique for the rat, since a similar injection does not induce arthritis in the mouse. However, it should be noted that high doses of pristane, if injected intraperitoneally, induces severe inflammatory disease course in mice, although the symptoms differ from PIA in rats and does not mimic RA (129).

PIA usually starts 10 days after immunisation and peaks at 20 days. The inflammation then gradually disappears before the relapsing chronic phase with new joint inflammation and cartilage erosion and deformity begins. PIA shares many clinical and subclinical features with RA, such as edema, infiltration of mononuclear and polymorphonuclear cells into the joint, pannus formation, and bone and cartilage destruction. Although the exact pathogenic mechanism of PIA is not clear, PIA is known to be αβ T-cell dependent (117) and can be adoptively transferred by activated $CD4^+$ T cells (130). There is however no evidence that B cells or antibodies play any pathological role in PIA (123). RF, α_1-acid glycoprotein (AGP), cartilage oligomeric matrix protein (COMP) and the proinflammatory cytokine IL-6 can be detected in rats with PIA. Similar to RA and CIA, PIA is regulated by both MHC and non-MHC genes (115, 116, 131).

2.1.3 Oil-induced arthritis (OIA)

The Incomplete Freund's adjuvant (IFA) has often been used to enhance immune response to antigens in different autoimmune disease induction protocols such as CIA. It was later unexpectedly discovered that IFA alone can induce arthritis in rats of DA strain (57, 117). Unlike pristane, which is a well-defined alkane molecule, IFA is a poorly defined mixture consisting of many hydrocarbon molecules of different molecular weights (132). In contrast to PIA, which is a chronic, relapsing disease model, OIA induces transient inflammation that usually starts 10 days after immunisation and lasts about 3 to 5 weeks (117). Other than these differences, OIA is very similar to PIA and leads to inflammatory, symmetrical and destructive polyarthritis (57). Histologic examination of the affected paws shows the typical features of inflammatory arthritis, including infiltration of inflammatory cells, synoviocyte hyperplasia, pannus formation, and bone and cartilage destruction. OIA depends on the polyclonal activation of T cells and can be adoptively transferred by activated T cells (123). Similar to RA in humans, OIA is regulated by both MHC and non-MHC genes (133).

2.1.4 Arthritis induced by other hydrocarbons

Hydrocarbon molecules other than pristane are also known to induce arthritis. Alkanes with 15 carbons or more induce arthritis; whereas shorter alkanes do not (134–136). In addition to the length of the hydrocarbon backbone, saturation of the hydrocarbon molecule, the presence of the halogen atom(s) and the presence of branched group(s) are known to affect the arthritogenicity of the hydrocarbon (135, 136). Among all the hydrocarbon molecules, squalene (SIA), and to a lesser extent hexadecane (HXIA) and heptadecane (HPIA), have been used to study arthritis in rats. Squalene is a cholesterol precursor and one single intradermal injection can induce joint-specific inflammation in the arthritis-susceptible rat strain DA (137, 138). Squalene-induced arthritis is T cell dependent and is regulated by both MHC and non-MHC genes (133).

2.1.5. Mycobacterial adjuvant-induced arthritis (Mbt-AIA)

Mbt-AIA is induced by an intradermal injection of heat-killed mycobacteria such as *Mycobacterium butyricum* or *Mycobacterium tuberculosis* in incomplete Freund's adjuvant. Mbt-AIA is monophasic and aggressive, and leads to severe systemic manifestations including splenomegaly and hepatomegaly. Although the pathogenic mechanism of Mbt-AIA is not clear, the heat shock protein 65 (HSP65)-derived peptide from mycobacteria is thought to be one of the immunogens (139). Similar to RA and other arthritis disease models, Mbt-AIA is regulated by both MHC and non-MHC genes (140).

2.1.6. Streptococcal cell-wall induced arthritis (SCWIA)

Components of the streptococcal cell wall have also been used to induce arthritis in rats (141). Arthritis is induced by an intraperitoneal injection of a suspension of group A streptococcal cell-wall fragments (141). The clinical disease is characterised by an acute inflammation in the distal joints, detected between days 1 and 4, and a chronic phase occuring approximately 3 weeks after immunisation with pannus formation, cartilage degradation, bone erosions and T cell infiltration (142). Administration of streptococcal cell-wall components into athymic inbred Lewis rats results in acute arthritis but not

chronic arthritis, suggesting that the acute phase of SCWIA is T cell independent whereas the chronic phase is T cell dependent (143). SCWIA mimics various clinical features of RA, including inflammation in peripheral joints, synovial pannus formation and synovial fibroblast proliferation.

2.2. Disease gene identification

The availability of rat models of arthritis with stable and reducible phenotypes has facilitated the genetic studies of arthritis. Different genome-wide strategies have been used in the past 20 or so years to map arthritis-associated loci, including F2 intercross, F1 backcross, advanced intercross lines (AILs) and heterogeneous stocks (HSs) (Figure 1). Initially, genetic linkage analysis is performed to identify QTLs that regulate different arthritis traits, such as disease onset, severity and antibody production (126, 131, 133, 140, 144, 145). The arthritis-regulating effect of the QTL can then be reproduced in a congenic strain (131, 145, 146). Once the disease phenotype is reproduced in a congenic strain, smaller subcongenic strains that still retain the disease phenotype can be generated to narrow down the arthritis-associated loci (147–150). The aim is to generate a congenic fragment comprising the minimum number of genes, so that it is possible to positionally identify the underlying causative gene(s) regulating the disease phenotype, and to characterise the pathological roles of these gene(s) (Figure 1).

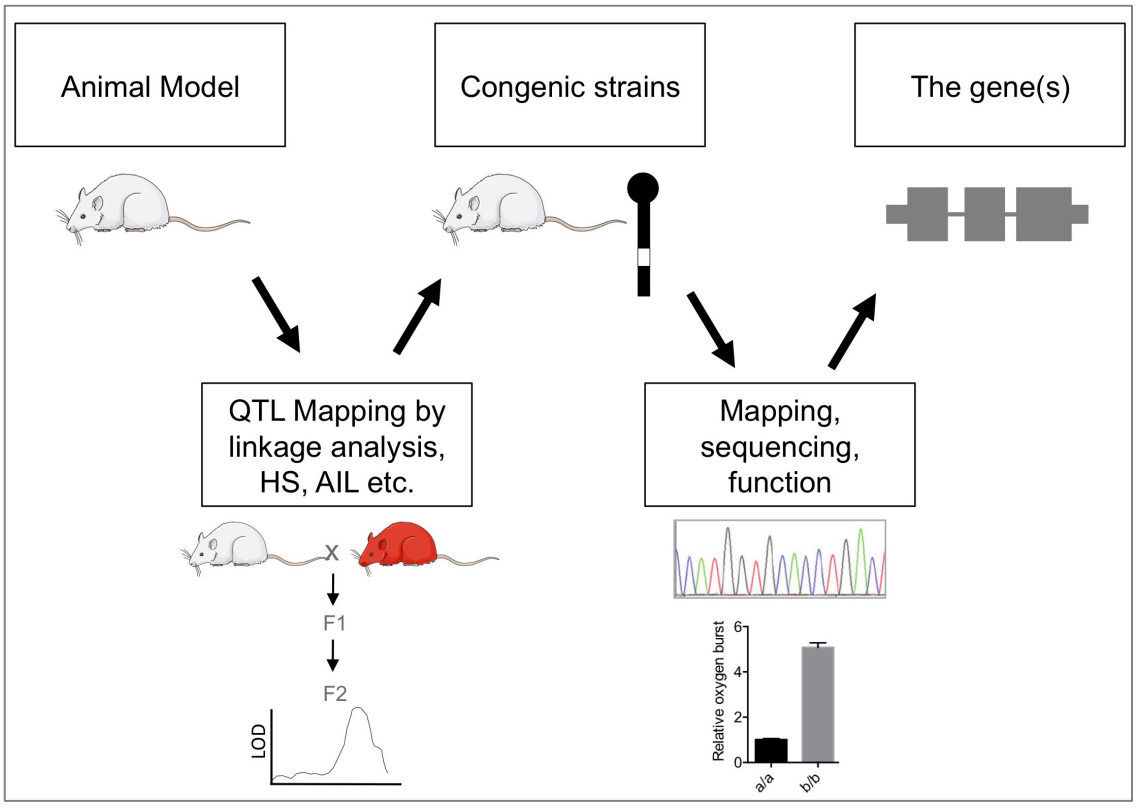

Figure 1: Strategy for positional identification of genes.

2.2.1 Arthritis-regulating QTL mapping: linkage analysis

The first stage in arthritis gene identification is genetic linkage analysis to map arthritis-regulating QTLs. The aim is to locate chromosomal regions associated with the regulation of arthritis phenotypes. The basis of the QTL mapping is that each animal in the linkage analysis has a unique genotype due to recombination events occurring during meiosis. Therefore, each animal needs to be genotyped using genetic markers that spread along the chromosomes in order to detect their parental origin. In addition, each animal is also evaluated for phenotypes of interest (which can be a disease trait). Linkage analysis is then performed to construct a genetic linkage map to detect loci linked to the phenotype and a measurement of the probability that loci are linked is calculated as the logarithm of the odds (LOD) score.

There are different methods of QTL mapping. The first method is to perform an F2 intercross between F1 hybrids of the two strains. The offsprings can then be used in genetic linkage analysis to identify arthritis-associated QTLs. The second method involves backcrossing of the F1 hybrids with one of the two parental inbred strains and the offsprings are used for linkage analysis. An F2 intercross has the advantage of giving more information concerning recessive alleles inherited from either of the two parental strains and also allows higher resolution mapping of genes with additive inheritance. A backcross analysis is more statistically powerful but identifies less QTLs (151).

Using PIA as an example, both F2 intercross and F1 backcross were successfully applied to identify QTLs in different chromosomes regulating different arthritis traits (131, 152). For example, an F2 intercross between arthritis-susceptible DA and arthritis-resistant E3 showed that *Pia2* and *Pia3* (loci on chromosome 4 and 6) regulate arthritis onset, *Pia4* (chromosome 12) regulates arthritis severity and joint erosion, and *Pia5* and *Pia6* (chromosome 4 and 14) are associated with arthritis chronicity (152). A later backcross analysis reproduced some of these findings and identified new QTLs *Pia10*, *Pia12*, *Pia13*, *Pia14*, and *Pia15* on chromosomes 10, 6, 7, 8, and 18, respectively (131). Similar genome-wide linkage analyses were used in CIA (126, 127) and Mbt-AIA (140). Most disease loci associate with both types of arthritis models, adjuvant-based (usually pristane and/or IFA) and antigen-based (usually type II collagen) (126, 145); however a few loci are implicated in only certain arthritis models. For example, *Cia4* regulates PIA and OIA but not CIA, whereas *Cia6* regulates OIA but not PIA and CIA (145), indicating specificity in the pathways in which some of these QTLs may operate.

In addition to F2 intercross and F1 backcross, another genome-wide QTL mapping strategy is HS. The rat HS is generated by intercrossing 8 inbred progenitor strains (ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N and WN/N) for ~60 generations (153) and has been used to map QTLs regulating different phenotypic traits (154). However, this strategy cannot be used to study arthritis QTLs because the inbred progenitor strains used to generate HS are resistant to arthritis (Tuncel and Holmdahl, unpublished). Similar limitations also occurred in HS mice (known as the Northport Stock) since the original inbred mouse strains used in the HS (namely A/J, AKR/J, BALB/CJ, LP/J, CBA/2J, C3H/HeJ, C57BL/6J and DBA/2J) lack the MHC class II $H2^q$ haplotype for presentation of

the immunodominant peptide of type II collagen (155). This was shown when the immunisation of the HS mice with CII induced almost no arthritis (156). To resolve this problem, the HS mice were crossed with an inbred strain, C57BL/10.Q, which has the arthritis-permissive $H2^q$ haplotype. These HS mice backcrossed to C57BL/10.Q were then successfully used to identify loci regulating different arthritis traits, such as disease onset, severity, incidence and antibody production (155, 157).

Another QTL-mapping approach is advanced intercross lines (AILs), which are lines generated by first crossing two inbred strains (for example, DA and PVG) to produce an F1 generation and then randomly intercrossing the progeny for many generations. Through random and repeated intercrossing, the rats accumulate new recombinations in every generation. In one group, rat AILs were used to study myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (MOG-EAE), a chronic relapsing model that mimics multiple sclerosis in rats (158–161). This AIL was generated by crossing EAE-susceptible DA and EAE-resistant PVG.1AV1 rat strains and was successfully used to identify and fine-map EAE-regulating QTLs (158–161). Another AIL panel GKxF344 was used to identify the locus regulating rheumatoid factor production in rats (162). AIL was also established in mice from CIA-susceptible DBA/1J and CIA-resistant FVB/N mouse strains that share the $H2^q$ haplotype. This AIL has been used for high-resolution fine mapping of QTLs controlling the onset, severity, T cell subset and antibody response of CIA (163).

2.2.2 From QTLs to genes: congenic strains and positional cloning

The second stage in gene identification is more laborious and takes longer time involving genotyping many generations and disease-phenotyping many rat strains. Once a QTL is identified, the goal is to firstly confirm the QTL effect in a congenic fragment, and secondly minimise the fragment to arrive at a region as small as possible, ideally containing only one (or a few) genes. Once this is achieved, further experiments can be performed to identify and analyse the functional roles of the disease causative gene(s).

Congenic strains are generated by transferring a specific chromosomal region from a donor strain to a recipient inbred strain. Initially, the donor strain is crossed to the recipient strain. The goal is then to replace, through many generations of backcrosses to the recipient strain, the ‘contaminating’ donor alleles with recipient alleles, with the exception of the disease-associated locus. Each backcross statistically reduces the ‘contaminating’ donor alleles by 50% and theoretically after ten generations of backcrossing, the resulting new strain will consist of ~99.8% recipient strain and can be considered a congenic strain. Since each generation takes about 3 months, this process of backcrossing can take almost 3 years. This process can however be shortened by the generation of ‘speed congenics’ so that only five to six, instead of ten, generations of backcrossing are needed. This is achieved by performing genome-wide analysis of genetic polymorphisms at each generation. Animals for the next generation of breeding are then selected by not only the presence of donor allele at the locus of interest but also by the absence of ‘contaminating’ donor alleles in other parts of the genome.

Once a congenic strain is established and the QTL disease phenotype is reproduced in the congenic strain, the next step is to minimize the size of the congenic fragment. Initially, congenic fragments are usually large stretching many megabases with tens or hundreds of genes and it is thus very difficult to pinpoint the causative genes. The congenic strain is screened for genetic recombinations. When a recombination is identified, smaller subcongenic strain can be generated and tested for the disease phenotypes. Once a smaller recombinant which still possesses the disease phenotype is identified, then we search for even smaller recombinants with fewer genes yet still possessing the disease phenotype. Finally, when the locus is reduced to the smallest size that is difficult to dissect any further by genetic recombination, in depth analysis of the locus is then performed to identify the disease causative gene(s) (if the locus consists of many genes) and to elucidate pathogenic mechanisms of the disease gene. This analysis may involve extensive DNA sequencing of the locus to identify all the genetic polymorphisms, expression analysis of the genes in the region to identify differentially-expressed genes, protein analysis by methods such as western blot or mass spectrometry, or functional assays such as promoter assays or transfection experiments. To obtain conclusive evidence of the functional consequence of any particular polymorphism, additional genetic manipulation of the candidate genes, such as transgenic or knockout technology, is often required.

2.2.3 Positional cloned genes

Over the past 20 or so years, different research groups have identified over 100 QTLs that contribute to various arthritis traits in rats (www.rgd.mcw.edu). These QTLs regulating different disease phenotypes including disease onset, severity and autoantibody production were identified in different models (CIA, PIA, OIA, AIA). Among all these QTLs, five underlying causative genes or gene clusters have so far been successfully positionally cloned (164). These genes are *RT1-B*, *Ltab-Ncr3*, *APLEC*, *Ncf1* and *Igl.* The genes *APLEC*, *Ncf1* and *Igl* will be briefly discussed in this section and studies on *RT1-B* and *Ltab-Ncr3* will be discussed in further depth in section 4.

Ncf1

Ncf1 (neutrophil cytosolic factor 1) is the first positionally-cloned gene in rats (165). It is a gene that is located inside the locus *Pia4,* which emerges in an F2 intercross between the arthritis-susceptible DA and the arthritis-resistant E3 (152, 165). *Pia4* regulates not only PIA, but also CIA, OIA, and HXIA. In order to identify the gene underlying the *Pia4* disease association, congenic strains with *Pia4* from the E3 genetic background introgressed into the DA background were generated and tested for arthritis. By testing different congenic strains that covered different parts of the *Pia4* locus, the associated region was gradually narrowed to 300-kb interval that is associated to arthritis protection (165). This 300-kb interval contained only two genes, *Ncf1* and *Gtf2i* (general transcription factor Iii). Only *Ncf1* is polymorphic with two non-synonymous single nucleotide polymorphisms (SNPs) at positions 106 and 153 of the protein and it was later shown that position 153 mediates arthritis resistance in rats (166). As part of the NADPH complex, NCF1 is involved in the production of reactive oxygen species (ROS) (167). The finding that arthritis-protective congenic rat showed an increased ROS production (165) was

surprising since the release of ROS is widely believed to be proinflammatory. This discovery in rats was further supported by the findings that a mouse with spontaneous mutation in the *Ncf1* gene produces undetectable ROS and develops enhanced arthritis (168). In humans, the *NCF1* region in human is more complicated, characterised by deletions, duplications and inversions (169). A case-control study showed that an increased copy number of *NCF1* is associated with arthritis protection (170). The effect of *Ncf1* is not arthritis-specific. For example, mutated *Ncf1* has been associated with EAE (168), spontaneous development of lupus (171) and also increased severity in mouse model of psoriasis (172) and gout (173).

APLEC

In addition to the *Pia4* locus, another PIA QTL that emerged from the F2 intercross was *Pia7* located on chromosome 4 (152, 174), which was found to also regulate OIA (133), and CIA (175). The QTL effect was reproduced in congenic strains (126, 131, 146, 176) and was positionally mapped to a 544-kb interval with the *APLEC* (antigen-presenting lectin-like receptor gene complex) (148, 149). This complex consists of lectin-like receptor genes, including *Mincle* (macrophage-inducible C-type lectin), *Mcl* (macrophage C-type lectin), *Dcar1* [dendritic cell (DC) activating receptor 1], *Dcir1-4* (DC immunoreceptor 1-4) and the Dectin pseudogene *Dectin2p* (DC-associated C-type lectin 2 pseudogene). Six of the seven genes in this complex were found to be differentially expressed in the lymph node and/or carry a missense or nonsense mutation in DA rats (148). It remains to be shown whether the effect of the *APLEC* locus is due to a haplotype with contributions coming from multiple interacting genetic polymorphisms, or is due to polymorphism(s) in a single gene exerting the QTL effect. In addition to arthritis, the *APLEC* polymorphisms also have other effects, including disease susceptibility and severity, and antibody response of MOG-EAE (177); and motor neuron survival after traumatic nerve root injury (178). In the mouse, APLEC has also been associated with arthritis (179), experimental colitis (180, 181), EAE as well as response to infection (182).

Igl

Rheumatoid factors were the first autoantibodies identified in RA and can be detected in serum long before disease onset (7). However, very little is known about the genetic control of RFs. In rats, linkage analysis on a (DAxE3) F_2 cohort identified three genetic loci that regulate RF production, namely *Rf1*, *Rf2* and *Rf3* (183). To study the effect of one of these three genetic loci, *Rf1*, a 6.7-Mb congenic strain was generated by introgressing *Rf1* from E3 onto DA background. However, it was found that this 6.7-Mb region undergoes little recombination and it is thus difficult to further positionally identify the underlying causative gene simply by generating smaller subcongenic fragments (162). In order to overcome this difficulty, the advanced intercross line (AIL) generated between GK and F344 rat strains was used, genotyped and studied for RF levels. Combining the data from both the congenic and AIL crosses, we concluded that the *Igl* locus is linked to the RF production (162). In addition, the congenic rat developed more severe ovalbumin-induced airway inflammation, a model of allergic bronchitis or asthma (162).

2.3 Advantages and limitations of animal models and the approach of linkage analysis and congenic strains

In order to advance our knowledge of rheumatoid arthritis, animal model is essential for a better understanding into the pathogenesis of the disease. This thesis describes how we use animal models to overcome limitations involved in human genetic studies to identify novel disease genes and to discover previously unknown disease mechanisms.

The main advantage of using experimental disease models is the possibility to perform *in vivo* functional studies in well-controlled genetic and environmental settings, which is not possible in humans. In particular, the rat provides a unique opportunity to study arthritis induced by oil adjuvants, which is one of the environmental risk factors of RA (10) (section 1.6). PIA is therefore an excellent model for studying the effect of adjuvants. PIA is highly reproducible with almost 100% incidence and induces a T-cell mediated, chronic relapsing disease course that closely mimics RA (116).

The possibility to isolate congenic fragments on a fixed genetic background allows us to discover natural genetic variants of importance. For example, the *NCF1* gene is usually not studied in human GWAS as a result of the complexity known in the human *NCF1* region but was discovered in congenic rat analysis to have an important role in arthritis (165, 170). Once a disease gene is identified, deep analysis of the pathological roles of the gene can then be performed using the congenic strain. For example, the association of the arthritis-protective DA.*Ncf1*^{E3} congenic rats with increased ROS production suggests an unexpected, protective role of ROS in autoimmunity. In addition, since many autoimmunity loci associate with multiple autoimmune diseases (184), congenic strains are highly useful to investigate the contributions of RA-associated genes to other autoimmune diseases. For example, *APLEC* polymorphisms was shown to regulate not only arthritis but also EAE (177); and *Igl* regulates ovalbumin-induced airway inflammation (162).

However, it is also important to consider the differences between experimental arthritis and RA in humans when interpreting findings from animal models. Firstly, disease induction differs between experimental arthritis and RA. Experimental arthritis is induced by intradermal injection at the base of the tail with relatively well-defined agents, such as hydrocarbon adjuvants and/or a particular antigen, such as type II collagen. The disease trigger in humans is not identified but could possibly include airborne pollutants, food, cosmetics and so on. In humans, the disease is also most likely induced differently from rats. Different administration routes are known to lead to different disease outcomes in rats (116) and experimental arthritis models are likely only reflecting specific types of RA mechanisms, depending on the immunisation agents. This can be useful to investigate a particular type of RA mechanism, but the disease course in a particular model might not mirror the complex disease course in humans. It is also important to consider any genetic differences between humans and rats. For instance, in the rat genome, there is only one *Ncf1* gene. In the human genome, there are several identical copies of *NCF1* due to segmental duplications (185), and an increased copy number of *NCF1* has been associated with lower susceptibility to RA in humans (170).

There are also drawbacks and limitations to use the linkage analysis and congenic approach to study disease association. Firstly, linkage analysis and congenic approach is performed in inbred strains and thus the ability to identify QTLs entirely depends on the allelic variants present in the genomes used. Secondly, positionally identifying a disease gene by isolating and narrowing down putative loci in congenic strains can take many years and requires a lot of animal breeding and screening. Thirdly, the success of positional cloning depends on identifying genetic recombinations. Both recombination-rich hotspots and coldspots exist in genomes (186) and thus some linked regions can be extremely difficult to split by recombinations. Transgenic and knockout technologies are attractive alternatives to study the functions of candidate genes, and new advances have enabled scientists to modify genes of any species, including rats. It is however important to note all genetic modifications are vulnerable to artefacts and could lead to different phenotypic outcomes (168, 187, 188). This could be due to regions flanking the targeted gene remaining of donor origin despite many generations of backcrossing to the host strain (189). It is also noteworthy that while targeted mutagenesis can be very useful for investigating specific hypothesis, it does not fully explain variation in complex traits. It has been suggested that single variants do not always entirely explain the genetic signals at a QTL and there can be multiple causal variants within a single QTL that contribute to the observed phenotypes (154). Even though positional cloning and functional assessment of genes in animal models is both time-consuming and expensive, it is more cost-effective and offers more functional insights than human genetic studies, for example, starting from large-scale GWAS.

3. MAJOR HISTOCOMPATIBILITY COMPLEX AND AUTOIMMUNE ARTHRITIS

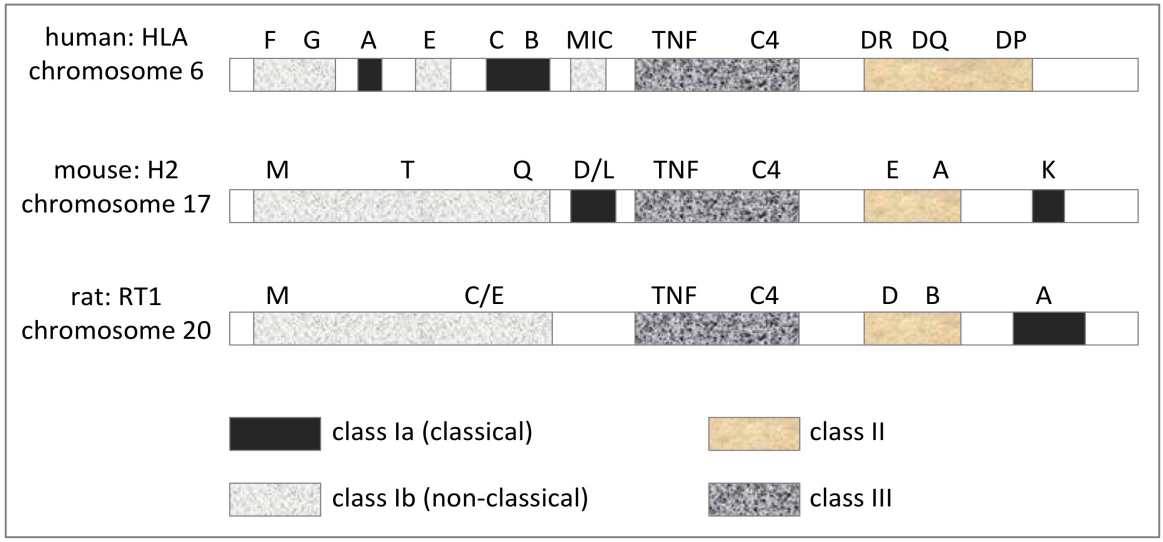

Figure 2: A comparison between the genetic organisation of the human (HLA), mouse (H2) and rat (RT1) major histocompatibility complex.

The ~4-Mb major histocompatibility complex (MHC) plays a key role in regulating immune response and is associated with susceptibility to different autoimmune and infectious diseases. The MHC region is located on chromosome 6 in humans, chromosome 17 in mice and chromosome 20 in rats (Figure 2). The MHC can be largely divided into three regions, the class I (MHC-I), II (MHC-II) and III (MHC-III) regions. The MHC-I and MHC-II regions contain genes encoding highly polymorphic MHC-I and MHC-II polypeptides that are involved in presentation of antigenic peptides to T cells. In humans, the MHC-I region includes genes such as *HLA-A*, *HLA-B* and *HLA-C,* and the MHC-II region includes genes such as *HLA-DP, HLA-DQ*, and *HLA-DR*. The class Ia molecules present intracellular antigens to cytotoxic $CD8^+$ T cells; whereas the class II molecules present antigens to $CD4^+$ T cells. The function of the class Ib genes are not clear. The MHC class III region contains an array of genes encoding mostly immune-related proteins, including the tumour necrosis factor (*TNF*) superfamily, component of the complement cascade, and molecular chaperones such as heat-shock proteins.

The MHC is the most gene-dense region in the genome. The MHC region contains ~230 known genes and pseudogenes. The average gene density of the MHC region is approximately 1 gene per 16 kb, which is higher than the genome average of 1 gene per 60 kb (190). The MHC region is extremely polymorphic (191), in particular for MHC-I and MHC-II molecules, and this is thought to be driven by the rapidly-changing needs of our immune system. Not surprisingly, the MHC region is associated with more diseases than any other regions in the human genomes and is linked to nearly all autoimmune diseases (192). Nevertheless, the functions of many of the genes in the MHC region are still unknown and it is estimated about 40% of the expressed genes have immune functions (190). Clustering of genes with similar immune functions is quite common in the MHC

region, for instance, in the classical MHC-II region where almost all the genes are immune-related. Proximity of genes might reflect the coevolution of functions or coexpression of transcripts. There is also an interesting difference in the proportion of pseudogenes in different MHC regions. For instance, there are very few pseudogenes in the MHC-III region; whereas there are many pseudogenes in the MHC-I and MHC-II region. It is also found that alternative splicing is enriched among MHC genes compared to non-MHC genes and alternative splicing seems to be haplotype-specific (193).

3.1 MHC class I

The structure of the MHC-I region differs quite significantly between rat, mouse and human. Firstly, the rodent MHCs are unique in the presence of centromeric MHC-I gene cluster proximal to MHC-II regions, called RT1-A in rats and H2-K in mice (Figure 2). Secondly, MHC-I genes in rats and mice also vary in copy numbers between different haplotypes (194). Thirdly, both rat and mouse MHC lack the *MIC* genes (*MICA* to *MICG*) present in human MHC-I. Lastly, the rat MHC-I RT1-A and RT1-C/E regions are distinctly different from mouse in various genomic features, such as gene numbers and organisation, suggesting independent evolution of the region after speciation of mouse and rat 16 to 23 million years ago (194). Telomeric to MHC-II and MHC-III, there are clusters of non-classical MHC-I genes which can be found in both humans and rodents. MHC-I molecule consists of three domains, namely α1, α2, and α3, and a non-MHC molecule β2 microglobulin unit is attached to the α1 domain. The transmembrane domain α3 anchors the MHC class I molecule to the cell membrane and the peptide-binding groove is located on the α1/α2 heterodimer. In humans, *HLA-A*, *HLA-B* and *HLA-C* encode the MHC class I heavy chains. Polymorphisms of the MHC-I proteins result in unique peptides binding to the peptide binding grooves of each protein.

MHC-I molecules present peptides of cytosolic origin. First, antigens are degraded by the proteasome. The resulting peptides are then transported from the cytosol to the endoplasmic reticulum (ER) via the transporter associated with antigen presentation (TAP) (195). Inside the ER, TAP forms the peptide loading complex (PLC) consisting of MHC-I molecules, ERp57, calreticulin and tapasin. Although peptides are trimmed before entering ER, peptides may be trimmed further by ER aminopeptidase associated with antigen processing (ERAAP) before binding with MHC-I (196). Peptide-MHC class I complexes are then transported to the plasma membrane via the Golgi for antigen presentation to $CD8^+$ T cells. In the ER, ER-associated protein degradation (ERAD) system transports unbound peptides back to the cytosol for further trimming or destruction by peptidases and the proteasome (197).

3.2 MHC class II

The MHC-II genes encode a number of peptide-binding proteins and proteins involved in the process of antigen loading. The organisation of the MHC-II region, both the gene content and order, are generally well-conserved in rats, mice and humans. Similar to MHC-I, there are duplicated modules in MHC-II. Among the 19 MHC-II genes in humans, 8 are pseudogenes. The number of pseudogenes differs between haplotypes (198). MHC-II itself

is a heterodimer consisting of an α chain and a β chain, each with two domains, α1 and α2, β1 and β2. The transmembrane domain α2 and β2 anchor the MHC-II molecule to the cell membrane and the peptide-binding groove is formed on α1 and β1 domains. The human MHC-II locus encodes three classical MHC-II proteins on the cell surface: HLA-DR, HLA-DQ and HLA-DP (mouse expresses I-A and I-E, rat expresses RT1-B and RT1-D). The MHC-II molecules are mainly constitutively expressed by professional APCs, such as dendritic cells, macrophages and B cells; and these MHC-II molecules present peptides to $CD4^+$ T cells. Polymorphic residues are found mostly in the β chains of HLA-DR, HLA-DQ and HLA-DP. The HLA-DR α chain is invariant, and the α chains of HLA-DQ and HLA-DP are partly polymorphic. The amino acid variants are mostly located in and around the peptide-binding groove of these MHC-II molecules, resulting in different peptides associated with different MHC-II alleles. Unlike MHC-I, MHC-II peptide binding groove is more open so that peptides can extend out of the MHC-II structure. This means MHC-II can bind to both longer peptides and also unfolded and native proteins (199).

Different from the ubiquitously-expressed MHC-I molecules, MHC-II molecules are expressed by professional APCs, such as dendritic cells, macrophages and B cells. In the ER, the MHC class II αβ dimers associate with the invariant chain (Ii) (200, 201). The Ii–MHC-II heterotrimers are then transported through the Golgi to the plasma membrane, where the heterotrimers are internalised by clathrin-mediated endocytosis and transported to antigen-processing compartments termed MHC class II compartments (MIICs). Here, Ii is degraded by resident protease such as cathepsin S and cathepsin L in the MIIC (202), leaving a residual class II-associated Ii peptide (CLIP) in the binding groove of the MHC-II dimer (203). The CLIP competes with other peptides in the MIIC to exclude low affinity, non-specific peptides from the binding groove of the MHC-II. In the MIIC, the HLA-DM regulate the exchange of the CLIP peptide for a specific peptide (204, 205) and the activity of HLA-DM is modulated by HLA-DO via an unknown mechanism. MHC class II molecules are then transported to the plasma membrane for peptide presentation to $CD4^+$ T cells.

3.3 MHC class III

The organisation of the MHC-III region is also conserved between rats, mice and humans with a few exceptions. First, at the centromeric end of the MHC-III region, there is a rodent-specific expansion of the butyrophilin-like (*Btnl*) genes. These genes expand independently (194), suggesting rapid evolution of these genes. Second, there is a rat-specific modular duplication of the *C4* complement gene and adjacent regions that is not found in humans and mice. The MHC-III region is the most gene-dense in the human genome, containing ~60 genes in ~700 kb with an average gene size of ~8.5 kb, compared to genome average of 27-45 kb (35, 206, 207). MHC-III region is very compact. While the average number of exons per gene (~11) and the size of the mRNA (~1.7 kb) is similar to the genome average, the average size of the MHC-III genes is significantly smaller than the genome average (MHC-III: 8.5 kb in humans, 7.7 kb in mice versus human genome average: >27 kb) (35, 207). MHC-III genes have shorter introns (MHC-III: 0.63 kb in humans, 0.58 kb in mice versus genome average: 4.7 kb in humans, 3.9 kb in mice) and shorter intergenic distances in MHC-III than genome average (35, 208). Several MHC-III

genes undergo extensive alternative splicing, for example *LST1* and *APOM*. The *LST1* gene is known to have at least 17 splice variants encoding for transmembrane as well as soluble LST1 in humans (209). Unlike the highly polymorphic MHC-I and MHC-II regions, the sequence variation of the MHC-III is actually comparable to the genome average (35). The MHC-III proteins have functions different from MHC-I and MHC-II and play wide-ranging roles such as acting as component of the complement system, cytokines, heat shock proteins and so on. Several of the MHC-III proteins that are more relevant to this thesis are briefly discussed here.

TNF is one of the most well-known MHC-III protein and was first discovered as a factor in serum in response to endotoxin that caused necrosis when injected into tumours (210). Mainly produced by monocytes and tissue macrophages, TNF plays a central role in triggering the inflammatory reactions of the innate immune system, such as inducing cytokine production and stimulating growth. TNF is initially produced as membrane-bound protein, and is released as a homotrimer upon cleavage by TNF-converting enzyme (TACE) and binds to one of the two receptors, TNFR1 or TNFR2. This initiates further signalling cascade leading to phosphorylation of NF-κB and activation of the p50-p65 subunit, which then increases transcription of other proinflammatory genes. Synthesis of TNF is tightly regulated because of its important proinflammatory functions.

The lymphotoxin genes (*LTα* and *LTβ*) are located next to *TNF* in the MHC-III region in the genome. Similar to TNF, LTα also binds to TNFR1 and TNFR2 as a homotrimer, and in addition, binds to the herpes virus entry mediator (HVEM). Furthermore, LTα forms a heterotrimer with LTβ and binds to the LTβR. Both LT and TNF are proinflammatory (211, 212), although it is generally believed that LT is not as strong and important as TNF. LT is essential for the development of secondary lymphoid organ, since LTα knockout mice have no lymph nodes, no Peyer's patches and highly disorganised spleens (213, 214). LTβ-deficient mice have similar but less drastic phenotype, and unlike LTα-deficient mice, retain cervical and mesenteric lymph nodes (215), which suggests a unique signalling role for $LT\alpha_3$. Thus, LT is important in the maintenance of lymphoid organs through its production by B cells, T cells and DCs.

The biological functions of LST1 proteins are largely unclear and it is also not known how many of the splice variants are translated into proteins. One of the transmembrane variants LST1/C have been shown to exhibit inhibitory effect on lymphocyte proliferation (209). Overexpression of the isoform LST1/A in human cell lines was shown to induce the formation of filopodia and microspikes at the cell surface (216) and was recently suggested to play a negative regulatory role on signal propagation in myeloid cells (217).

3.4 Regulation of MHC class II expression by CIITA

MHC-II expression is tightly regulated at the level of transcription by MHC class II transactivator (CIITA). CIITA is the master regulator of MHC class II expression, and is recruited by the enhanceosome to the X1, X2 and Y box elements in the MHC-II genes (218). CIITA then coordinates the recruitment of other factors involved in chromatin modification and remodelling such as CREB-binding protein (CBP) and p300. CIITA also

coordinates the recruitment of factors for transcription initiation and elongation, such as transcription factor IIB (TFIIB) and TFIID (218). There are four *CIITA* isoforms in humans (pI, pII, pIII and pIV) (219) and three *Ct2a* isoforms in the mouse and rat (pI, pIII and pIV), each with a cell-specific promoter (type I, III and IV). The type I promoter drives the CIITA expression by conventional dendritic cells and interferon-γ activated macrophages. The type III promoter drives CIITA expression by B cells and activated human T cells, as well as plasmacytoid dendritic cells. The type IV promoter is important for CIITA expression by cortical thymic epithelial cells and in cells of non-haematopoietic origin such as fibroblasts upon interferon-γ induction (220, 221).

3.5 MHC class II and autoimmune arthritis

Since the identification of the link between HLA and RA half a century ago (34) which was further defined by the 'shared epitope hypothesis' (36) (as described in section 1.5), many groups have examined the association between SE and RA in different cohorts and different subgroups of RA. SE is significantly associated with increased risk of developing RA, especially for ACPA-positive RA (222–228). However, it is important also to note that not every RA patient carries SE alleles and SE alleles do not necessarily lead to development of RA. This suggests that genes other than *HLA-DRB1* also contribute to disease aetiology. Different studies have detected RA association signals from other MHC regions, from both within the class II region (*DPB1, DOB, DQA, DQB*), as well as class I region (*HLA-C*, *HLA-B*) and class III region. (229–232). Recent meta-analyses confirmed and refined the SE to positions 71 and 74 and extends to position 11 (and 13) in individuals with ACPA-positive RA (3). These studies identified disease association in HLA-A (position 77), HLA-B (position 9) and HLA-DPB1 (position 9) (3, 4). Interestingly, the strongest association signal was detected at HLA-DRB1 amino acid position 11 (or 13), which is located outside the previously described shared-epitope region but within the antigen-binding groove. Only after controlling for position 11 in HLA-DRB1, positions 71 and 74 in HLA-DRB1 and other association signals were detected by stepwise conditional analyses. It is not clear however whether position 11 or 13 is the main driving influence due to the physical proximity of the two positions. Similar disease association signals were detected in ACPA-positive RA cases among Chinese and Korean populations (233). The exact functional roles of the amino acid variants in an MHC-II molecule on disease development remains to be clarified, although the location of the amino acid variants within the peptide-binding grooves suggests that these polymorphisms might have an impact on antigenic presentation to T cells, either during early thymic development or during peripheral immune responses.

In rats, the strong genetic association of MHC genes on chromosome 20 with the development of experimental arthritis has been shown in different linkage analyses. In the F2 intercross between disease-susceptible DA and relatively resistant F344 rat strains, it was found that the MHC locus is associated with both CIA (denoted as *Cia1*) (127) and Mbt-AIA (denoted as *Aia1*) (140). A backcross between the disease-susceptible DA and disease-resistant E3 rat strains similarly showed that the MHC regulates PIA (denoted as *Pia1*) and CIA (126, 131). Congenic rat strains differing only in the MHC locus similarly demonstrated that MHC regulates different models of experimental arthritis, including CIA, PIA and OIA (126, 131, 145, 234–236). The importance of MHC is also shown in analysis

of congenic mice strains that only strains expressing certain MHC class II such as A^q but not A^b (expressed by the C57BL/6) conferred susceptibility to arthritis (237–239). This thesis describes how we utilised a panel of MHC-II congenic rat strains to study the pathogenic mechanisms of the MHC-II in experimental arthritis.

3.6 MHC class III and autoimmune arthritis

In addition to the MHC-II genetic contributions to RA, multiple studies have suggested independent genetic association of MHC-III genes with RA development (232, 240–247). Given the important immunoregulatory roles of the MHC-III proteins as discussed in section 3.3, some of them have also been described as candidate genes for RA. The most well-known candidate gene in the region is *TNF* (247). Different SNPs in the *TNF* gene (for instance, at positions -308, -238, +489) have been investigated for disease association and a recent meta-analysis showed that TNF-308 is associated with joint damage in patients with RA (247). Both monoclonal anti-TNF antibody and TNFR fusion proteins have been developed to target TNF for RA treatment (248, 249). Other candidate genes include *LTA* (*lymphotoxin α*) (250, 251), *LTB* (*lymphotoxin β*), *LST1* (*leukocyte specific transcript 1)*, *NCR3 (natural cytotoxicity triggering receptor 3)* (252), and *AIF1* (*allograft inflammatory factor 1*) (241, 246). The importance of lymphotoxins in experimental arthritis was shown when it was found that blocking *LTB* resulted in altered development of CIA in mice (253, 254). A monoclonal antibody Pateclizumab targeting $LT\alpha_3$ and $LT\alpha_1\beta_2$ has been developed and is under initial clinical trial in RA patients (255). In addition, significant elevation in expression of *LTB, NCR3* and an isoform of *LST1* have been found in blood and synovium from RA patients (250, 252) and increased expression of *AIF1* was found in RA mononuclear cells (241, 246). However, the high gene density, extreme polymorphism and the strong LD across the MHC have made this region very difficult to investigate for disease association (256, 257) and so far evidence showing the direct contribution of genetic polymorphism in the MHC-III in pathogenesis of RA is lacking. This thesis describes how we made use of congenic rat strains isolated in the MHC-III region to show natural genetic polymorphisms in the MHC-III regulates experimental arthritis.

3.7 CIITA and autoimmune arthritis

Given the known strong link between MHC-II and development of RA, the master regulator of MHC-II genes, *CIITA*, has also been studied to access the impact of quantitative difference of MHC-II expression on arthritis development in both humans and rodents. It was first identified in a genetic analysis of an advanced intercross line in rats that polymorphisms in the 5' flanking region of *C2ta* explain strain-dependent differences in expression of MHC-II molecules (258). Congenic rat strain with the *C2ta* polymorphisms not only had a lower MHC-II expression but also developed milder experimental autoimmune encephalomyelitis (EAE), a chronic relapsing model of MS (259). The experimental findings in rats led to further investigation of *C2TA* in humans and a single nucleotide polymorphism in the 5' flanking region of type III promoter of *CIITA* has associated with the risk of developing MS, RA and myocardial infarction (258). *CIITA* disease association has since then been shown in different patient cohorts, in not only RA (260, 261) and MS (262, 263), but also T1D (264), celiac disease (265) and systemic lupus

erythematosus (SLE) (266, 267). Nevertheless, this disease association could not be reproduced in some populations (268–270). Several SNPs located in different parts of *CIITA* have been studied for disease association, including -168A/G polymorphism, rs3087456 in the type III promoter of *CIITA,* an intronic SNP rs8048002, and rs4774 which is a missense mutation resulting in an amino acid change from glycine to alanine (258, 260, 263). The rs3087456 is the most investigated for disease association in humans. This thesis describes how we used a *C2ta* congenic mouse strain to study the effect of natural genetic variants in the type I promoter of *C2ta* on MHC-II expression and the subsequent development of autoimmune diseases, including arthritis.

4. PRESENT INVESTIGATIONS

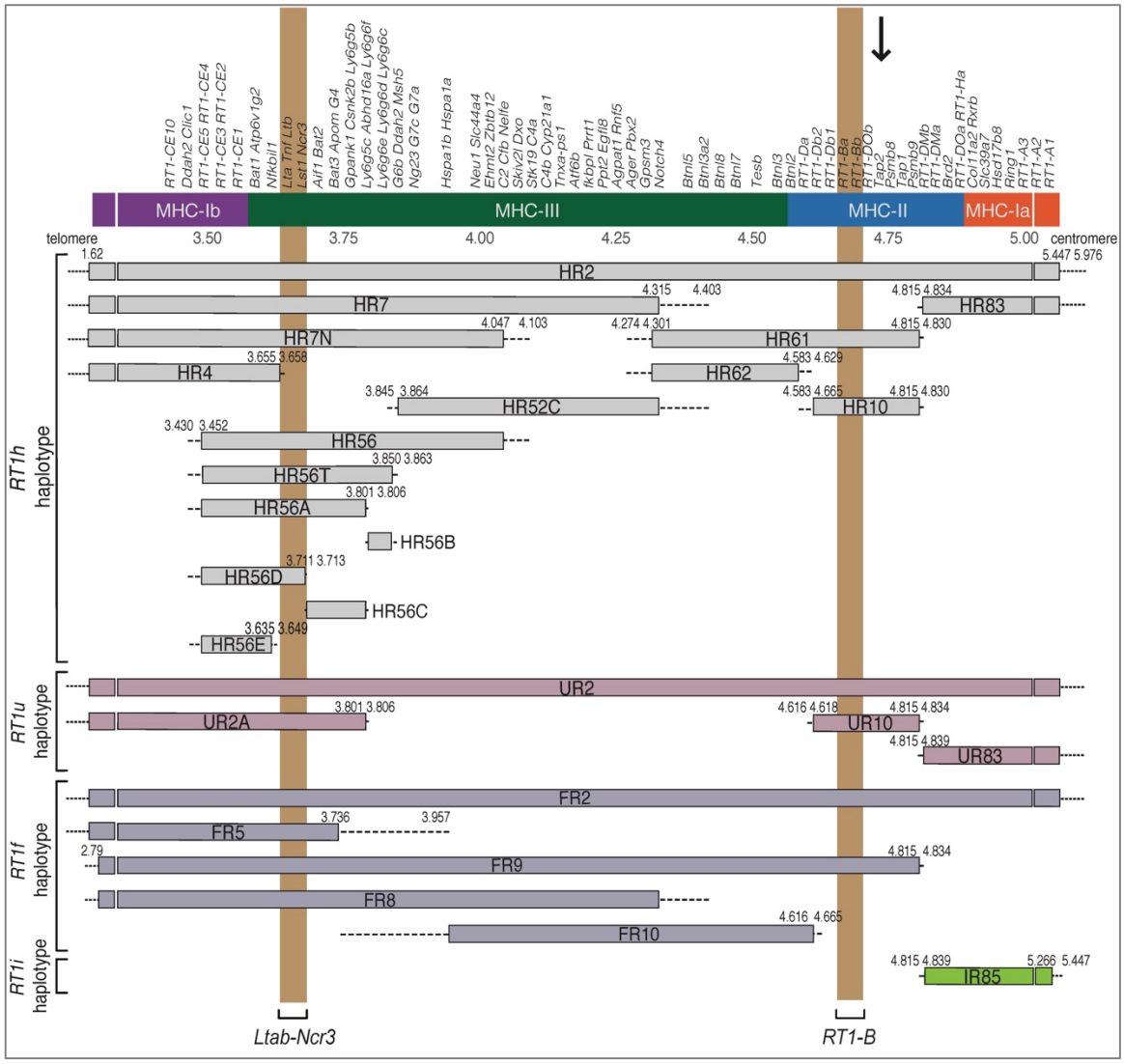

Figure 3: Overview of the main MHC congenic recombinant strains discussed in this thesis. Studies I and II describe the regulation of arthritis by the MHC-II *RT1-B* genes (shaded in brown). Studies IV and V describe the regulation of arthritis by the MHC-III *Ltab-Ncr3* genes (shaded in brown). Study III describes how *Tap2* (indicated by an arrow) in MHC-II influences negative selection and T cell lineage commitment.

4.1 Study I

Positional Identification of RT1-B (HLA-DQ) as Susceptibility Locus for Autoimmune Arthritis

Since the strong link between MHC-II and RA was known more than 40 years ago, little is known about the precise underlying causative polymorphisms. Recent results from genome-wide association studies refined the association to six different amino acids in HLA-DRB1, HLA-A, HLA-B and HLA-DPB1 (3, 4). The strong genetic association of MHC genes in experimental arthritis was also shown in multiple linkage analyses (127, 131, 140, 152) and reproduced in congenic rat strains (131, 145, 236).

In this study, we examined the genetic association of the MHC-II genes using a panel of congenic strains mapped to the MHC-II region. Three congenic strains were generated by introgressing different inbred rat strains, namely KHW ($RT1^{h}$ haplotype), AS2 ($RT1^{f}$) and E3 ($RT1^{u}$), onto a DA ($RT1^{av1}$) background. We induced pristane-induced arthritis (PIA) in these rats and assessed their arthritis development. Two of these congenic strains, namely DA.1HR61 ($RT1^{h}$) and DA.1UR10 ($RT1^{u}$) developed milder acute PIA than wt DA controls ($RT1^{av1}$); whereas DA.1FR9 ($RT1^{f}$) developed enhanced acute PIA. In order to pinpoint the underlying causative genes within the 0.2-Mb mapped QTL (called *Tcs2*), we examined the coding variants of the genes within *Tcs2* and correlated the coding variants with disease severity. We identified that *RT1-Ba* and *RT1-Bb*, the rat orthologues of the HLA genes, *HLA-DQA* and *HLA-DQB*, to be the main candidate genes determining arthritis susceptibility. Blocking RT1-B with an antibody against RT1-B resulted in significantly milder PIA in all strains, further supporting a role of *RT1-B* in PIA. We found that the RT1-B was differentially expressed on both macrophages and dendritic cells. However, the variation in expression levels of RT1-B does not explain the difference in arthritis severity between the strains. We then compared the peptide repertoire of RT1-B between different strains; and showed that the peptide repertoire of RT1-B differs substantially between strains. The binding pockets of the arthritis-protective congenic strains DA.1HR61 and DA.1UR10 prefer binding with glutamic acid; whereas arthritis-promoting DA.1FR9 binds preferentially to hydrophobic amino acids. The peptide binding data is further supported by the fact that arthritis-protective strains share amino acid variants in the peptide-binding RT1-B P1 pocket.

In summary, we positionally identified a 0.2-Mb interval in the rat MHC-II region that regulates the onset and severity of PIA; and our genetic and functional findings on the four MHC-II haplotypes provide strong evidence that amino acid variations in *RT1-B* may explain this arthritis regulation.

4.2 Study II

Class II Major Histocompatibility Complex–Associated Response to Type XI Collagen Regulates the Development of Chronic Arthritis in Rats

The aims of this study were to characterise the immune response at different stages of pristane-induced arthritis (PIA) in rats to joint-specific cartilage antigens, such as type II collagen (CII) and type XI collagen (CXI), and to identify the underlying genetic association in the MHC-II region. This is achieved by generating and assessing arthritis development in recombinant congenic rat strains (haplotype $RT1^{f}$) covering different parts of the MHC region.

We first assessed the susceptibility to CII-induced arthritis (C_{II}IA) and CXI-induced arthritis (C_{XI}IA) in congenic rat strains DA.1FR8, DA.1FR9 and DA.1FR10, and DA control rats. For C_{II}IA, while DA.1FR9 rats developed milder arthritis, DA.1FR8, DA.1FR10 and DA rats developed severe arthritis. This shows that the ~0.2-Mb MHC-II region contributes to susceptibility to C_{II}IA. Almost the opposite occurs in C_{XI}IA. While DA.1FR9 rats developed severe C_{XI}IA. DA.1FR8, DA.1FR10 and DA rats developed mild arthritis. Taken together, susceptibility to C_{II}IA and C_{XI}IA was linked to haplotypes $RT1^{av1}$ and $RT1^{f}$, respectively. Having mapped the collagen response to the ~0.2-Mb MHC-II region, we next evaluated T cell and antibody responses to CII and CXI at different stages of PIA. After pristane injection, both DA.1F ($RT1^{f}$) and DA ($RT1^{av1}$) developed T cell and antibody responses to CII, but not CXI. However, in chronic PIA, rats developed stronger reactivity to CXI, not CII, and such reactivity is restricted to $RT1^{f}$ haplotype. Genetic sequence analysis of the four MHC class II genes, *RT1-Ba*, *RT1-Bb*, *RT1-Da* and *RT1-Db1*, found a number of coding variants in the peptide-binding domains of RT1-Ba but no polymorphisms in RT1-D. This strongly supports an association of *RT1-B* genes with CXI immunity. In order to assess whether the collagen reactivities seen in PIA is also found in RA, we determined the proportion of RA patients with antibody responses to CII and CXI. We showed that 12% of RA patients developed specific IgG response to CXI, 6% to CII and 6% to both.

In summary, this study demonstrates the development of MHC-II-associated reactivity towards CXI in chronic arthritis suggesting a role of CXI autoreactivity in the development of chronic disease.

4.3 Study III

Natural Polymorphisms in Tap2 Influence Negative Selection and CD4⁚CD8 Lineage Commitment in the Rat

This study aims to address the impact of natural genetic polymorphisms in both MHC-I and MHC-II regions on MHC expression and CD4:CD8 lineage commitment using both the outbred heterogeneous stock (HS) rats and inbred recombinant congenic strains generated in the MHC region.

Using the HS rats, we mapped genome-wide QTLs which are associated with variation in the MHC-I and MHC-II protein expression and the CD4:CD8 T cell ratio. A total of ten QTLs were identified across the genome. Among these QTLs, we identified a region spanning 4.1-9.7 Mb on chromosome 20. Such region overlaps with the MHC region that regulates both CD4:CD8 T cell ratio and MHC expression.

In order to refine this region that contributes to this phenotypic variation, we generated a panel of MHC-recombinant congenic strains derived from AS2 ($RT1^{f}$), KHW ($RT1^{h}$), E3 ($RT1^{u}$) and BI ($RT1^{i}$) on a DA ($RT1^{av1}$) background. The congenic panel includes congenic strains mapped to MHC-I (*Tcs1*) and MHC-II (*Tcs2*) regions. Phenotyping of the MHC-I and MHC-II congenic strains revealed that 282-kb *Tcs1* regulates MHC-I expression and CD4:CD8 T cell ratio; and the 206-kb *Tcs2* regulates both MHC-I and MHC-II expression, and the ratio of CD4:CD8 T cells. The effect of *Tcs2* on MHC-I expression and the T cell numbers was in fact due to interactions between *Tcs1* and *Tcs2;* and we studied this interaction effect using congenic strains isolated in the MHC-I and MHC-II region made possible by recombinations occurring between *Tcs1* and *Tcs2*. By correlating the sequence variants of the genes within *Tcs2* with variation in MHC-I expression, we mapped the interaction effect to *Tap 2* (transporter associated with antigen processing 2) in the MHC-II region and the classical MHC-I genes *RT1-A* in the MHC-I region. This study confirmed the previously known phenomenon, class I modification (cim), which reduced the expression of TAP-A linked RT1-A molecules if associated with TAP-B. In addition, this study identified a novel phenomenon which we named ‘inverse’ cim. Inverse cim reduced the expression of TAP-B linked RT1-A molecules when linked with TAP-A (Figure 4). Genetic variations in *Tap2* have previously been shown to alter the peptide repertoire of MHC-I molecules which then influence the antigenicity of the MHC-I molecules (272, 273). This study further shows that a restricted peptide repertoire on MHC-I molecules reduces negative selection of CD8 single-positive T cells in the thymus.

In summary, we show how combination of a particular allelic variant of *Tap2* and a particular allelic variant of *RT1-A* genes could influence negative selection and lineage commitment of T cells in rats.

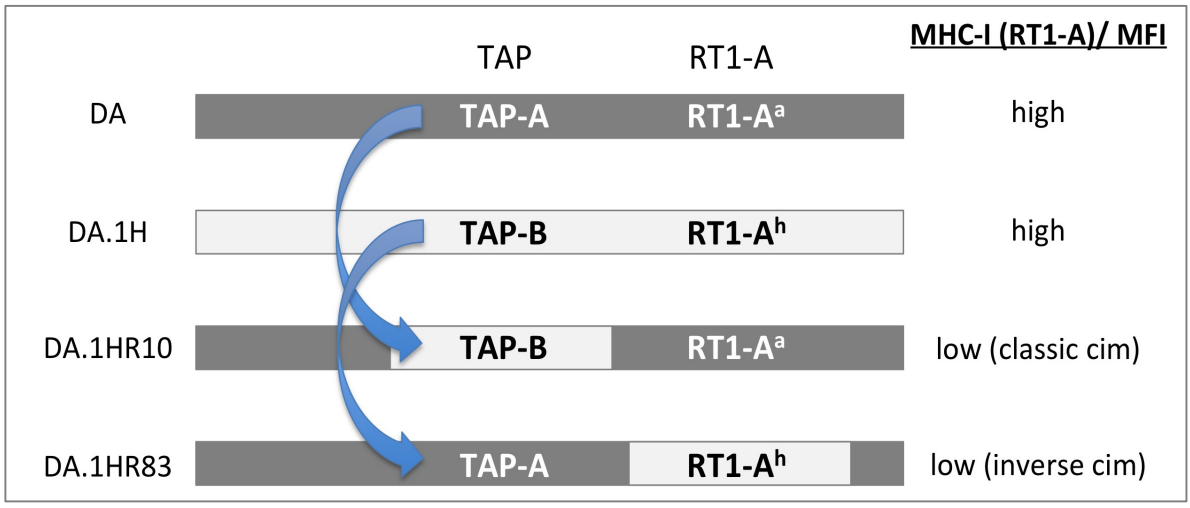

Figure 4: Summary figure for class I modification (cim) and inverse cim. The classical cim reduced the expression of TAP-A linked RT1-A molecules if associated with TAP-B. The inverse cim reduced the expression of TAP-B linked RT1-A molecules when linked with TAP-A.

4.4 Study IV

Conserved 33-kb haplotype in the MHC class III region regulates chronic arthritis

Recent results from genome-wide association studies confirmed the association between classical MHC-II alleles and RA (3, 4). In addition, different studies have suggested that genes in the other parts of the MHC region, such as MHC-III, also contribute to arthritis development (232, 240–246), as discussed in section 3.6. However, this disease contribution has been difficult to show owing to the high gene density, extreme polymorphism and the strong linkage disequilibrium across the MHC (256).

In study I, we showed that the *RT-1B* genes in MHC-II regulate the acute phase of pristane-induced arthritis (PIA) (147). In this study, using MHC-III congenic strains, we showed that there is a second arthritis QTL in the non-classical MHC-Ib and/or the MHC-III region. By generating and disease-phenotyping a panel of ten subcongenic strains mapped to these regions, we further narrowed down this QTL to a 32.7-kb interval, denoted *Ltab-Ncr3*, in the telomeric end of the MHC-III region comprising five genes, *Lt*α*, Tnf, Ltb, Lst1* and *Ncr3*. We showed that *Ltab-Ncr3* determines not only the onset and severity, but also the chronicity of PIA. By performing the reciprocal adoptive transfer of *in vivo* pristane-primed T cells between the congenic and DA rats, we demonstrated that *Ltab-Ncr3* regulates the priming but not the effector phase of arthritis.

We found that the *Ltab-Ncr3* region is rather resistant to recombinations, as evident from the absence of recombination in this region in over 8000 rats genotyped. SNPs information in the *Ltab-Ncr3* region in non-related wild rats also supports the existence of a conserved haplotype in *Ltab-Ncr3*. Significant correlation in the expression of the *Ltab-Ncr3* genes provides further support that interaction of these genes may be of importance in maintaining these genes clustered together as a conserved haplotype.

By performing a number of *in vitro* and *in vivo* assays in the congenic and control rats, we showed that polymorphisms in *Tnf* do not contribute to the *Ltab-Ncr3* haplotype effect. We assessed the level of expression of *Ltab-Ncr3* genes in the arthritis-protective congenic DA.1HR56D (haplotype $RT1^{h}$) and arthritis-prone control DA ($RT1^{a}$); and also in other arthritis-prone congenic DA.1FR9 ($RT1^{f}$) and DA.1UR2A ($RT1^{u}$). We showed that higher *Ltb* and *Ncr3* expression, lower *Lst1* expression and the expression of a shorter isoform of *Lst1* correlate with reduced arthritis severity (Figure 5). We examined the expression of *LTB, LST1* and *NCR3* genes in the whole blood from a cohort of 32 RA patients and 92 healthy controls. Similar to the findings in the DA.1HR56D arthritis-protective congenic rats, patients with mild RA (DAS28 ≤ 3.2) also showed higher *NCR3* expression and lower *LST1* expression than patients with severe RA (DAS28 > 5.1).

In summary, we identified a 33-kb conserved haplotype in the MHC-III that determines the onset, severity and chronicity of experimental arthritis; and haplotype-specific differences in both gene expression and alternative splicing correlates with arthritis development. These data demonstrate the importance of a conserved haplotype in the regulation of complex diseases such as arthritis.

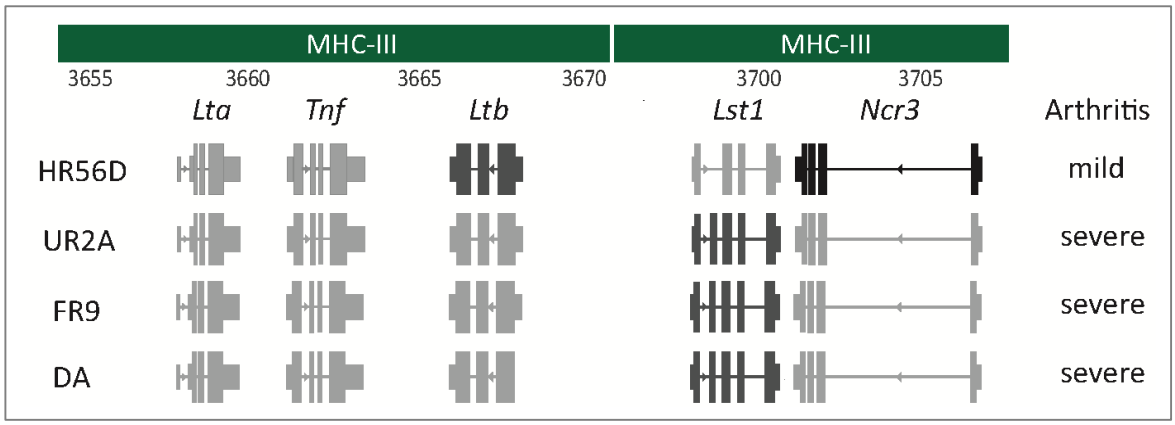

Figure 5: Higher expression of *Ltb* and *Ncr3,* lower expression of *Lst1*, and the expression of a shorter splice variant of *Lst1* correlate with reduced arthritis severity in rats.

4.5 Study V

MHC class III Ltab-Ncr3 haplotype regulates adjuvant-induced but not antigen-dependent autoimmunity.

In study IV, we identified a 33-kb conserved haplotype *Ltab-Ncr3* in the MHC-III that determines the onset, severity and chronicity of pristane-induced arthritis in rats. In study V, we evaluated this new genetic association with arthritis using different adjvuvant- and antigen-induced arthritis models. In addition, we used models for multiple sclerosis for comparison and dissect the MHC-III mediated mechanisms of importance for antibody and T cell responses to antigens.

Using the MHC-III recombinant congenic strains, we showed that the *Ltab-Ncr3* haplotype also regulates oil-induced arthritis (OIA), which is induced by the immunisation of incomplete Freund's adjuvant (IFA). Both macroscopic scoring and histology analysis showed that the MHC-III congenic rat strain DA.1HR56D developed milder OIA than DA controls. DA.1HR56D rats are similarly protected in arthritis models induced by other hydrocarbon oils, such as hexadecane, heptadecane, squalene and arlacel. Adoptive T-cell transfer experiment showed that this arthritis-protective effect operated during the priming of T cells. Interestingly, *Ltab-Ncr3* did not regulate autoimmune diseases induced with tissue-derived antigens emulsified in IFA, such as collagen-induced arthritis (CIA) and myelin-oligodendrocyte-glycoprotein-(MOG-) induced experimental autoimmune encephalomyelitis (EAE). The *Ltab-Ncr3* haplotype also showed no effect on antibody or T cell response to tissue antigens such as type II collagen.

To date, all the positionally cloned arthritis genes in rats (section 2.2.3 and study I, II) have been shown to regulate both PIA (and OIA) and CIA. *Ncf1 and APLEC* were shown to regulate PIA, OIA and CIA (126, 149, 165); and *RT1-B* (128, 147) regulates both PIA and CIA. To our knowledge, the *Ltab-Ncr3* haplotype is the first fine-mapped locus that regulates only oil adjuvant induced arthritis models. In contrast to the pronounced effect of *Ltab-Ncr3* on the priming of arthritogenic T cells in the oil adjuvant models, there was no such effect in CIA model or antigen-specific *in vitro* restimulation assay. One likely explanation is that the nature of T cell activation as a result of adjuvants exposing endogenous antigen *in vivo* is different from the one caused by the immunodominant effect of an administered exogenous antigen. We previously showed that addition of an immunogenic cartilage protein, such as CII, to IFA leads to a qualitative different disease, compared to the disease induced by IFA alone (120). This is also supported by our unpublished observations that passive transfer of CIA by CII-reactive T cells is not as effective as adoptive transfer of oil adjuvant-primed T cells. All these data suggest that the mechanisms involved in the priming of T cells with no exogeneous antigen in OIA (which is regulated by *Ltab-Ncr3*) is different from T cells triggered after immunisation of immunodominant self antigens, such as CII in CIA or MOG in EAE (and is not regulated by *Ltab-Ncr3*).

In summary, we showed that the 33-kb *Ltab-Ncr3* haplotype in the MHC-III regulates oil adjuvant arthritis models but not autoimmune diseases triggered through immunisation with

tissue-derived antigens. The *Ltab-Ncr3* haplotype seems to control mechanisms involved in the early events of an autoimmune response.

Table 3: Summary of *Ltab-Ncr3* regulation in different disease models.

Disease models	*Ltab-Ncr3* disease phenotype
Pristane-induced arthritis	protective
Oil-induced arthritis	protective
Hexadecane-induced arthritis	protective
Heptadecane-induced arthritis	protective
Squalene-induced arthritis	protective
Arlacel-induced arthritis	protective
Collagen-induced arthritis	no effect
rSCH-induced EAE	no effect
MOG-induced EAE	no effect

4.6 Study VI

Effects of C2ta genetic polymorphisms on MHC class II expression and autoimmune diseases.

MHC-II has been strongly associated with many autoimmune diseases including rheumatoid arthritis (RA) and multiple sclerosis (MS). Its expression is controlled by the class II transactivator (CIITA in human and C2TA in mouse), which is regulated by cell-specific promoters (I, III, IV) as described in section 3.4. As discussed in section 3.7, genetic variation in *CIITA* has been linked to susceptibility to autoimmune disorders in humans (258, 260–267). In rodents, genetic variants in type III promoter of *C2ta* has been studied in a congenic setting and shown to be associated with experimental autoimmune encephalomyelitis (EAE), a chronic relapsing model of MS (259). Mice with a knockout in type IV promoter however developed collagen-induced arthritis (CIA) with comparable severity (274).

In this study, we examined the effect of natural genetic variants in type I promoter of *C2ta* on MHC-II expression and autoimmune diseases. We achieved this by isolating a *C2ta* congenic fragment from the donor strain 129X1/SvJ onto a recipient C57BL/6N.Q background such that the congenic mice expressed the H2-Aq gene, facilitating the studies of the effect of *C2ta* in different experimental models of RA and MS (237, 275–277). We demonstrate that an allelic variant in the type I promoter of *C2ta* resulted in an increased expression of MHC-II on macrophages and conventional dendritic cells in both spleen and peripheral blood. MHC-II upregulation subsequently resulted in increased antigen presentation to T cells and increased T cell activation. This MHC-II expression difference however did not alter susceptibility of these mice to different experimental models of multiple sclerosis (MOG_{1-125} protein- and MOG_{79-96} peptide-induced EAE), or rheumatoid arthritis (CIA and human glucose-6-phosphate-isomerase$_{325-339}$-peptide-induced arthritis). This is similar to the findings in study I that MHC-II expression difference on APCs did not correlate with the arthritis severity in the MHC-II congenic rat strains. Therefore, this and other studies (147, 259, 274) seem to suggest that quantitative difference in MHC-II plays a relatively minor role in the regulation of autoimmune diseases triggered by exogenous immunisation, such as EAE and CIA.

In summary, we address the role of an allelic variant in the type I promoter of *C2ta* in MHC-II expression and autoimmune diseases; and demonstrate that upregulation of MHC-II expression and subsequent antigen presentation as a result of *C2ta* polymorphisms does not necessarily have a strong impact on autoimmune diseases.

5. ACKNOWLEDGEMENTS

Surprisingly, it does not seem to be that long ago when I first joined the lab as a pre-PhD student. Time flies and I would like to take this opportunity to thank everyone who has been part of this memorable PhD journey.

5.1 My supervisors and coauthors

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B2-416

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B2-418

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B2-422

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